Comparison of plasma virus loads among individuals infected with hepatitis C virus (HCV) genotypes 1, 2, and 3 by quantiplex HCV RNA assay versions 1 and 2, Roche Monitor assay, and an in-house limiting dilution method

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 187–192 Vol. 35, No. 1
0095-1137/97/$04.00+0

Comparison of Plasma Virus Loads among Individuals Infected with Hepatitis C Virus (HCV) Genotypes 1, 2, and 3 by Quantiplex HCV RNA Assay Versions 1 and 2, Roche Monitor Assay, and an In-House Limiting Dilution Method

A. HAWKINS,[1] F. DAVIDSON,[2] AND P. SIMMONDS[1]*

Department of Medical Microbiology, University of Edinburgh, Edinburgh, EH8 9AG,[1] and Edinburgh and South East Scotland Blood Transfusion Service, Edinburgh, EH3 9HB,[2] United Kingdom

Received 6 August 1996/Returned for modification 4 October 1996/Accepted 18 October 1996

The accuracy of different methods for the quantitation of hepatitis C virus in plasma was measured with samples from individuals infected with different genotypes and by using RNA transcripts of predetermined concentrations. Highly reproducible results were observed upon repeat testing of samples by both the original version of the Chiron branched-DNA (bDNA) assay (Quantiplex RNA assay; bDNA-1) and the currently available version (Quantiplex HCV RNA 2.0 assay; bDNA-2). A greater variability was observed in the Roche Monitor assay (correlation coefficient of 0.537, compared with 0.942 and 0.964 for the bDNA-1 and bDNA-2 assays, respectively). Significant differences in the efficiency of detection of genotypes 1, 2, and 3 were observed for the bDNA-1 and Roche Monitor assays, whereas the bDNA-2 assay and nested PCR at limiting dilution were able to quantify genotypes with equal sensitivity. By quantifying RNA transcripts of different genotypes, the sensitivities of the Roche Monitor assay for sequences of the type 2 and type 3 transcripts were estimated to be 11 and 8% of those achieved for genotype 1. When correction factors based upon these results and those from quantitation of circulating viral RNA sequences in samples from blood donors were used, the genotype-specific differences in virus load in samples from blood donors were no longer observed, consistent with previous studies with corrected values from the bDNA-1 assay. These results suggest that many of the previous studies evaluating the effect of genotype and virus load on the response to interferon using methods such as the Roche Monitor assay and other competitive PCR methods require reinterpretation. Differences in efficiency of quantitation should be taken into account in future investigations of the relationship between genotype and virus load.

Quantitation of hepatitis C virus (HCV) RNA sequences in plasma and more recently in liver has become a valuable diagnostic tool for the pretreatment evaluation of patients undergoing treatment with interferon and in the subsequent monitoring of their response (8). For example, several studies have described a decreased likelihood of achieving a long-term response to interferon treatment in individuals with a high pretreatment virus load in plasma (1, 12, 19–22, 28). This difference was shown to be independent of other pretreatment variables that also influenced response, such as severity of liver disease and virus genotype (19–21, 28).

Several studies have found that the virus load of patients infected with type 1b was higher than in patients infected with type 2a or 2b, and it has been argued that this difference may therefore, at least in part, account for the increased probability of achieving a long-term response with genotypes 2 and 3 (15, 17, 22, 28). Clearly, such analyses require that the assays used to determine virus load are equally sensitive for each genotype, but there is evidence that this was not the case for the original branched-DNA (bDNA) assay (Quantiplex HCV RNA assay; Chiron Corporation [referred to as bDNA-1 in this study]). Quantitation by the bDNA-1 assay of known amounts of RNA transcripts revealed a twofold reduction in the efficiency of detection of type 3 transcripts compared with those of type 1 (5). This and subsequent work (9) led to a recent proposal to correct raw data from the bDNA-1 assay by a factor of 2 for type 3 infections and by a factor of 3 for type 2 infections (14). Quantitation of genotypes 4, 5, and 6 was similar to that of genotype 1 (9).

Using these corrected values for the bDNA-1 assay, we recently found that there was no difference in median virus loads between any of the six genotypes in HCV-infected blood donors from different geographical regions (25). A similarly designed study found no difference in virus loads among genotypes 1 to 3 among patients with chronic hepatitis under assessment for interferon treatment (14). The implication of these findings is that the observed differences between genotypes in response to treatment are independent of virus load, although virus load may remain an independent predictor of response.

The currently available version of the bDNA assay (Quantiplex HCV RNA 2.0 assay [referred to as bDNA-2]) has been redesigned to allow equivalent quantitation of all genotypes (9). In the current study, we have measured the accuracy of quantitation of different HCV genotypes with this assay and compared it with that of the bDNA-1 assay, a commercially available competitive PCR system (Roche Monitor), and an in-house method based upon limiting dilution PCR with nested primers from the highly conserved 5′ noncoding region (5′NCR). These results have enabled a more detailed evaluation of the interrelationship between virus load and ge-

* Corresponding author. Mailing address: Department of Medical Microbiology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, United Kingdom. Phone: 44 131 650 3138. Fax: 44 131 650 6531. E-mail: Peter.Simmonds@ed.ac.uk.

TABLE 1. Quantitation of HCV RNA in plasma samples in four assays

Genotype (*n*)	Virus load[a]	bDNA-1	bDNA-2	Roche Monitor	Limiting dilution
1 (31)	Median	3.28	3.35	0.98	2.4
	Minimum	<0.35	<0.2	0.1	0.11
	Maximum	23.59	23.51	6.80	49
2 (29)	Median	0.96	3.59	0.28	2.4
	Minimum	<0.35	<0.2	<0.001	<0.001
	Maximum	12.04	32.75	19	24.5
	Ratio to type 1[b]	0.29	1.0	0.29	1.0
	P value[c]	0.003	0.906 (NS)	0.022	0.917 (NS)
3 (31)	Median	0.85	1.09	0.09	2.4
	Minimum	<0.35	<0.2	<0.001	<0.001
	Maximum	20.67	71.04	3.71	34
	Ratio to type 1	0.26	0.33	0.09	1.0
	P value	0.015	0.459 (NS)	<0.001	0.850 (NS)

[a] Virus load is expressed in 10^6 copies of RNA/ml.
[b] Ratio of median virus load to that of genotype 1 samples.
[c] Probability of differences in virus load between genotypes occurring by chance (Mann-Whitney *U* test [one-way analysis of variance]). Significant differences ($P < 0.05$) are underlined. NS, not significant.

notype and their relative importance to disease severity and in predicting response to treatment.

MATERIALS AND METHODS

Samples. Ninety-one plasma samples from HCV-infected, PCR-positive Scottish blood donors were analyzed for HCV RNA levels. Samples were repeatedly reactive on screening for antibodies to HCV with a third-generation screening assay (Ortho; Abbott), confirmed positive with RIBA-2 or RIBA-3 supplementary assays and PCR positive with a nested PCR based upon amplification of sequences from the 5′NCR (4, 18). Samples were aliquoted and stored at −40°C and were only frozen and thawed twice prior to testing. Consecutive samples were selected to provide suitable numbers of each genotype (31 type 1 [25 type 1a, 6 type 1b], 29 type 2 [all type 2b], and 31 type 3 [all type 3a]). HCV genotype was determined by restriction fragment length polymorphism analysis of sequences amplified from the 5′NCR (7).

Three commercial assays for the detection of HCV RNA were used: the Quantiplex HCV RNA assay (bDNA-1), the Quantiplex HCV RNA 2.0 assay (bDNA-2), and the Roche Monitor assay. These assays were performed strictly according to the manufacturer's instructions. Unamplified HCV RNA was detected in the bDNA-1 and bDNA-2 assays by hybridization to a labelled probe and amplification of the detection signal. The Roche Monitor assay is based upon reverse transcription and amplification of the HCV RNA with primers from the 5′NCR in the presence of a competitor RNA derived from a virus of genotype 1. Both the competitor and the sample DNA were detected by hybridization to a probe in a colorimetric assay.

For the in-house limiting dilution method (23, 24), viral RNA was extracted from 100 μl of plasma by incubation with proteinase K (1 mg/ml) and sodium dodecyl sulfate (0.55%) in the presence of polyadenylic acid (40 mg/ml) and purified by phenol-chloroform extraction. HCV RNA was precipitated in 70% ethanol and resuspended in 25 μl of diethylpyrocarbonate (DEPC)-treated water. Five microliters of this solution was reverse transcribed and amplified by nested PCR with primers specific for the 5′NCR. Limiting dilution of cDNA was then carried out in a series of 10-fold steps, which allowed quantification to within 1 log. The quantitation was further refined by the addition of a specific volume of cDNA to a number of replicate PCRs which would give a Poisson distribution of positive and negative samples and therefore allow the concentration of HCV cDNA to be determined. The previously established efficiency of 5% for the reverse transcription step was assumed in this assay (30).

Quantitation of HCV RNA transcripts. HCV transcripts of all three genotypes were provided by J. Detmer, Chiron Diagnostics, Emeryville, Calif.). The methods used for their synthesis and quantitation have been described elsewhere (5, 9). Briefly, to assess the quality of the preparations, HCV RNA transcripts were electrophoresed on 1.5% formaldehyde gels and scanned on an Ambis 4000 radioanalytic imager (Ambis, Ino, San Diego, Calif.). The preparations of the HCV RNA transcripts used in the study contained less than 3% free nucleotides and were composed of at least 80% full-length transcripts. Three independent analytical methods were used to quantify the transcripts. These included phosphate determination (6), measurement of A_{260}, and hyperchromicity analysis (27). Preparations of HCV RNA transcripts were measured in triplicate by each of the three methods, and quantification results agreed within 10%. Transcript RNA was diluted in DEPC-treated water ($N \times 10^5$ and $N \times 10^6$ transcripts) or a buffer containing 10 mM Tris, 1 mM EDTA, and 100 mM NaCl for the $N \times 10^7$ transcripts before analysis and stored at −70°C until use. Transcripts were quantified in the bDNA-2 and Roche Monitor assays.

RESULTS

Quantitation of HCV RNA in four different quantitation assays. Plasma samples were obtained from a total of 91 HCV-infected blood donors who were viremic as determined by a nested PCR with primers from the 5′NCR (4, 18). HCV genotypes were identified by restriction fragment length polymorphism analysis of amplified sequences (7). The study group was selected to include approximately equal numbers of each of the three common genotypes in Western Europe and comprised 31 individuals infected with type 1 (25 type 1a, 6 type 1b), 29 infected with type 2b, and 31 infected with type 3a.

Fresh plasma samples were aliquoted to ensure that all quantitative assays were carried out with samples that had been frozen and thawed only twice. HCV RNA was quantified with the bDNA-1 and bDNA-2 assays, the Roche Monitor assay, and an in-house quantitation method based upon limiting dilution of cDNA (Table 1). A total of 25 samples were negative in the bDNA-1 assay, with a cutoff of 0.35×10^6 copies of RNA/ml (27%), while 17 (19%) were negative in the bDNA-2 assay (cutoff sensitivity of 0.2×10^6 copies of RNA/ml). Among these, 17 samples were negative in both assays (1 type 1, 5 type 2, and 6 type 3) and 8 were negative in the bDNA-1 assay only (2 type 1, 3 type 2, and 3 type 3). In the Roche Monitor assay and limiting dilution assays, six samples (four type 2, two type 3) were below the cutoff (approximately 1,000 copies of RNA/ml). Samples below the cutoff for each assay were assigned the virus load of the cutoff. This approximation did not affect nonparametric methods used to analyze the results.

There was a significant correlation between assays for the measurement of virus load measured for each (Table 2). For

TABLE 2. Correlation between tests for quantitation of HCV RNA in type 1, 2, and 3 samples

Genotype and test	Correlation with[a]:			
	bDNA-1	bDNA-2	Roche Monitor	Limiting dilution
Type 1				
bDNA-1	1			
bDNA-2	0.961	1		
Roche Monitor	0.795	0.802	1	
Limiting dilution	0.729	0.744	0.612	1
Type 2				
bDNA-1	1			
bDNA-2	0.870	1		
Roche Monitor	0.637	0.662	1	
Limiting dilution	0.637	0.557	0.571	1
Type 3				
bDNA-1	1			
bDNA-2	0.903	1		
Roche Monitor	0.784	0.856	1	
Limiting dilution	0.755	0.712	0.632	1
All types				
bDNA-1	1			
bDNA-2	0.866	1		
Roche Monitor	0.764	0.698	1	
Limiting dilution	0.682	0.711	0.548	1

[a] Spearman's rank correlation coefficient (all values, $P < 0.001$).

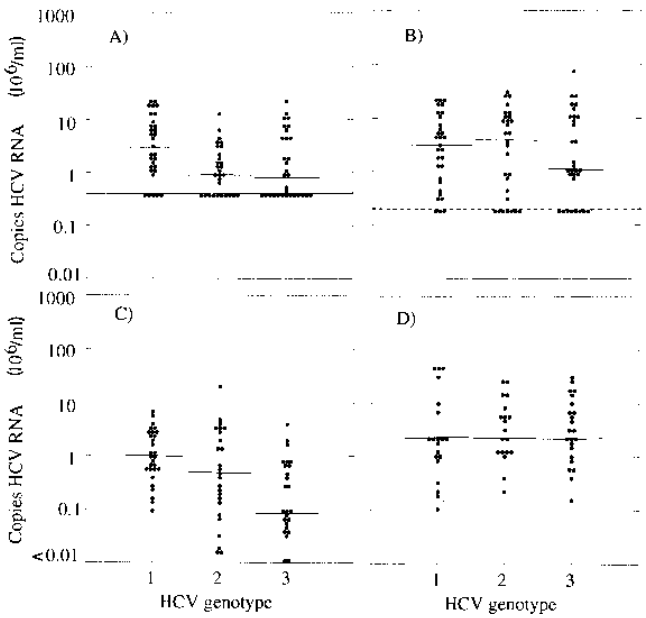

FIG. 1. Range of HCV RNA levels in plasma samples from blood donors infected with genotypes 1, 2, and 3 as detected by bDNA-1 (A), bDNA-2 (B), Roche Monitor (C), and limiting dilution (D) quantitative assays. Cutoff values for the bDNA-1 assay (0.35×10^6 copies of RNA/ml) are indicated by a solid line; cutoff values for the bDNA-2 assay (0.2×10^6 copies/ml) are indicated by a dashed line. Median values for each genotype are indicated by short solid lines.

example, over the range of quantitations of the bDNA-2 assay ($>0.2 \times 10^6$ copies of RNA/ml), positive correlation coefficients were observed upon comparison with the bDNA-1, Roche Monitor, and limiting dilution methods for quantitation. Similarly, nonparametric tests of correlations produced a high correlation coefficient upon pairwise comparison of each test (all values, $P < 0.001$ [Table 2]). The best correlation for all genotypes was between the results of the bDNA-1 and bDNA-2 assays, with a correlation coefficient of 0.866 (Table 2). This was also observed when virus loads in samples of different genotypes were analyzed separately (Table 2). Correlation coefficients for virus load among type 2 samples were lower than those observed for the type 1 or type 3 samples in all assays.

One factor that influences how well test results correlate is the reproducibility of the assays. To investigate this, we tested between 7 and 27 samples in duplicate in the four assays with separate aliquots of sample and, where possible, with different manufacturers' batches of reagents (Table 3). The most reproducible assay was the bDNA-2 assay, with a high correlation coefficient upon repeat testing of 20 samples (0.939) and a low standard deviation of ± 0.052 $\log_{10}$ values (95% confidence interval, approximately 26%). The least reproducible assay was the Roche Monitor assay, with a correlation efficient of 0.769 and a standard deviation of ± 0.311 $\log_{10}$ value (95% confidence interval, approximately 400%) upon repeat testing (Table 3). The corresponding values for the bDNA-1 and limiting dilution assays were 0.942 ± 0.1022 and 0.941 ± 0.184, respectively.

TABLE 3. Replicate testing of samples in quantitative assays

Assay	No. of replicates	Log_{10} variance[a]	Spearman rank correlation coefficient
bDNA-1	22	0.1022	0.942
bDNA-2	20	0.052	0.964
Roche Monitor	7	0.316	0.537
Limiting dilution	9	0.184	0.941

[a] Expressed as a standard deviation about a $\log_{10}$ mean value.

Virus load and genotype. In each assay, a wide range of virus loads was observed among donors infected with each genotype (Table 1 and Fig. 1). Median values calculated for these distributions differed between different quantitation assays. For example, among the type 1-infected donors, median values of 3.28, 3.35, 0.98, and 2.4 were observed for the bDNA-1, bDNA-2, Roche Monitor, and limiting dilution methods, respectively (Table 1). According to the bDNA-2 and limiting dilution assays, virus loads were similarly distributed between other genotypes, with median values of 3.59 and 2.4 for type 2 samples and 1.09 and 2.4 for type 3 samples, respectively. Using Wilcoxon's signed-rank test (Mann-Whitney U test), we observed no significant difference in virus load between donors infected with types 1, 2, and 3 (Table 1) when samples were analyzed with the bDNA-2 or limiting dilution assays.

In contrast, significant differences in virus loads between genotypes were observed with the other assays. In the Roche Monitor assay, median virus loads for types 2 and 3 were 0.28 and 0.09, 3.3 and 10 times lower than the values calculated for type 1 samples ($P = 0.022$ and $P < 0.001$, respectively), while there was no difference between types 2 and 3 ($P = 0.183$). Virus loads measured for the bDNA-1 assay were also different between genotypes; the type 2 and 3 samples were 3.3 and 4 times lower, respectively, than the type 1 samples ($P = 0.024$ and $P = 0.015$). The differences in virus load between genotypes in the bDNA-1 assay can in this case be attributed to the previously described reduced sensitivity of the bDNA-1 assay for type 2 and 3 sequences (see below).

The ratios of virus load measured in the bDNA-1, Roche

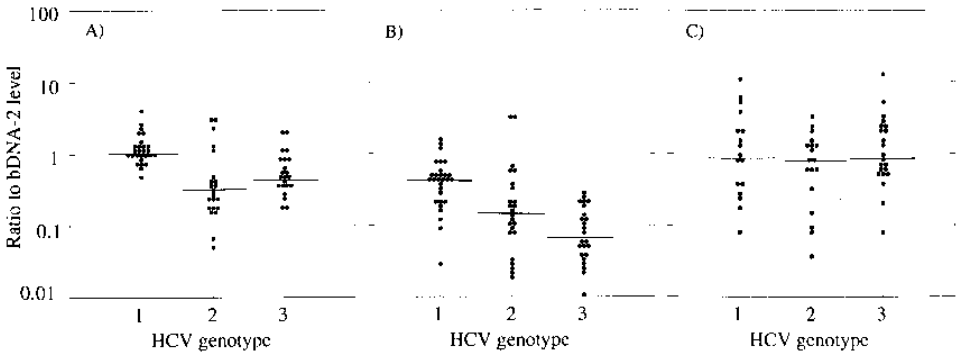

FIG. 2. Comparison of quantitation of genotypes 1, 2, and 3 by the bDNA-1 (A), Roche Monitor (B), and limiting dilution (C) assays expressed as ratios to levels determined in the bDNA-2 assay. Median values for each genotype are indicated by short solid lines.

Monitor, and limiting dilution assays to that measured by the bDNA-2 assay were calculated (Fig. 2 and Table 4). The scatter in values of the ratios reflects both the variance observed for each assay upon testing of duplicates and differences in the efficiency of detecting RNA sequences in the two assays. A particularly wide range of ratios were observed upon comparison of the Roche Monitor assay with the bDNA-2 assay (Fig. 2B), while the smallest range was observed upon comparison of the bDNA-1 and bDNA-2 assays (Fig. 2A). The ratios for two of the tests (Roche Monitor and bDNA-1) varied, depending upon genotype, with type 2 and 3 samples showing ratios and median ratios consistently lower than those observed for the type 1 samples (in each case, $P < 0.001$). In the extreme case, type 3 samples in the Roche Monitor assay showed a median ratio approximately eightfold lower (0.063) than that for type 1 (0.3). In contrast, no significant difference in ratio was observed upon comparison of limiting dilution with the bDNA-2 assay (ratios of 0.85, 0.74, and 0.76 for genotypes 1, 2, and 3, respectively).

Quantitation of HCV RNA transcripts. Three different concentrations of HCV RNA of HCV types 1, 2, and 3 were assayed in the Roche Monitor and bDNA-2 assays to investigate whether these tests differed in their sensitivity for sequences of different genotypes (Table 5). These were diluted to the operating ranges of the quantitative assays as described in Materials and Methods. The Roche Monitor assay was relatively inefficient at detecting RNA transcripts of all three genotypes. Type 1a sequences were detected with an efficiency of approximately 13.5%, whereas quantitation of the type 2b and 3a transcripts was even further from the expected values (1.5 and 1.1% efficiency, respectively). The type 2 transcript was detected with an efficiency 9-fold lower than that of type 1, while the efficiency of detection of type 3 was reduced by a factor of 12-fold. The bDNA-2 assay detected transcripts from each of the three genotypes with much higher efficiency. For type 1 transcripts, the mean sensitivity was 88% of the expected value, while for type 3 transcripts, the average was 60% of the input number of sequences. The type 2 transcripts were detected with an efficiency of approximately half that expected at the two lower dilutions.

TABLE 4. Efficiency of detection of genotype 1, 2, and 3 sequences relative to that of the bDNA-2 assay

Genotype (*n*)	Efficiency of detection	bDNA-1	Roche Monitor	Limiting dilution
1 (31)	Median	1.04	0.43	0.850
	Minimum	0.5	0.028	0.085
	Maximum	2.73	1.53	10.9
2 (29)	Median	0.25	0.15	0.744
	Minimum	0.054	0.001	0.037
	Maximum	1.36	3.4	3.18
	Ratio to type 1	0.22	0.34	0.86
	P value[a]	<u>≤0.001</u>	<u>≤0.008</u>	0.502 (NS)
3 (31)	Median	0.52	0.063	0.76
	Minimum	0.173	0.009	0.088
	Maximum	1.14	0.26	3.03
	Ratio to type 1	0.46	0.15	0.90
	P value	<u>≤0.001</u>	<u>≤0.001</u>	0.986 (NS)

[a] Probability of differences in virus load between genotypes occurring by chance (Mann-Whitney *U* test [one-way analysis of variance]). Significant differences ($P < 0.05$) are underlined. NS, not significant.

DISCUSSION

This study used a range of quantitative assays to measure virus load in individuals infected with different HCV genotypes. Even small genotype-specific differences in the sensitivity of these assays would have a significant compounding effect upon statistical analysis of the influence of these and other variables on disease severity and response to treatment. Although few studies have systematically addressed this issue, it has been reported that the now superseded bDNA-1 assay showed a significantly reduced sensitivity for genotypes 2 and 3 (5, 9). As a short-term measure to correct for this difference, it was suggested that virus loads measured by this assay in type 2 infections should be multiplied by 3 and those of type 3 should be multiplied by 2 (14). This study confirmed the difference in sensitivity of this assay for these genotypes (Table 4). Whereas the bDNA-1 and bDNA-2 assays measured similar virus loads in type 1 samples (median ratio, 1.04), the ratio between the bDNA-1 and bDNA-2 assays for type 2 was 0.22 (the expected value based upon the multiplication factor above would be 0.33), and that between for type 3 was 0.46 (expected value, 0.5).

A more satisfactory solution to the use of multiplication factors was the modification of the capture and target probes to remove their genotype dependence. The currently available version 2 of the assay (Quantiplex HCV RNA 2.0 assay) had been shown to quantify transcripts of HCV types 1 to 6 with similar efficiencies (9), and this was confirmed experimentally with RNA transcripts in the current study (Table 5).

Although we have no a priori knowledge of the actual virus

TABLE 5. Measurement of absolute efficiency of quantitation assays with RNA transcripts of genotypes 1, 2, and 3

Genotype	No. of input copies[a]	No. of copies of RNA/ml by[b]:	
		bDNA-2	Roche Monitor
1	4.4×10^5	7.1×10^5	0.28×10^5
	4.4×10^6	3.1×10^6	0.71×10^6
	1×10^7	0.33×10^7	0.18×10^7
2	4.4×10^5	2.9×10^5	0.071×10^5
	4.4×10^6	1.0×10^6	0.12×10^6
	1×10^7	0.39×10^7	0.0017×10^7
3	5.1×10^5	5.0×10^5	0.068×10^5
	5.1×10^6	2.3×10^6	0.07×10^6
	1×10^7	0.36×10^6	0.0047×10^7

[a] Transcripts were diluted in DEPC-H_2O, except for those at the 1×10^7 concentration, which were diluted in Tris buffer.

[b] The mean sensitivities (number of copies of RNA per milliliter) of the assays for genotypes 1, 2, and 3 were as follows: genotype 1, bDNA-2, 0.882; Roche Monitor, 0.135; genotype 2, bDNA-2, 0.44; Roche Monitor, 0.015; genotype 3, bDNA-2, 0.6; Roche Monitor, 0.011.

load in the plasma samples from blood donors, equal efficiencies of detection of sequences of different genotypes were implied by the observation of similar virus loads among those infected with the three genotypes (Table 1). Similarly, no significant differences in virus load between genotypes were observed with the bDNA-1 assay once the correction factors were applied (Table 1 and data not shown). Finally, quantitation based upon limiting dilution also found similar virus loads between genotypes (Table 1) and median ratios to the bDNA-2 assay values of 0.85, 0.74, and 0.76 for genotypes 1, 2, and 3, respectively (Table 4). The comparability between the results of these three tests argues strongly that no significant differences in virus load exist between genotypes, consistent with previous larger surveys of blood donors and patients (13, 25) and that quantitation by the (corrected) bDNA-1, bDNA-2, and limiting dilution assays is not influenced by sequence variation of HCV.

These results contrast strongly with those of the competitive assay investigated in the current study (Roche Monitor). Significantly lower levels of viremia were observed among samples collected from blood donors infected with genotypes 2 and 3 compared with those infected with genotype 1 (Table 1). The magnitude of this difference was comparable to that a previous study of hemophiliacs infected with genotypes 1 to 4 (2). Based upon the comparison with other quantitative assays in this study, we propose that the apparent differences in median virus load between genotypes determined with the Roche assay resulted from unequal quantitation, rather than reflecting true biological differences between them.

Further evidence for genotype dependence of the Roche assay comes from quantitation of known amounts of HCV RNA transcripts of different genotypes (Table 5). Whereas the bDNA-2 assay showed efficiencies of detection ranging from 44 to 88% for transcripts of different genotypes, the Roche Monitor assay showed generally much reduced levels, particularly for genotypes 2 and 3 (Table 5). Quantitation of type 2 sequences was approximately 11% that achieved for type 1 sequences, while type 3 sequences were detected with an efficiency of 8% compared with that of type 1 sequences. We are currently carrying out a more detailed investigation of the transcript quantitation in the Roche Monitor assay; however, these preliminary results do provide approximate correction factors that might be applied to the results from clinical specimens in a manner analogous to those proposed for the bDNA-1 assay (14). If the correction factors are applied to the Monitor results in the current study (i.e., multiplying virus load by a factor of 9 for type 2 samples and by a factor of 12 for type 3 samples), the previously observed differences in virus load between genotypes become nonsignificant (type 2 median ratio to type 1, 2.6; $P = 0.124$; and type 3 median ratio to type 1, 1.09; $P = 0.524$).

The reduced sensitivity of the Roche assay for sequences of genotypes 2 and 3 probably originates from mismatches between the PCR primers and the target HCV. The assay is based upon competition with the quantitation standard, which shows a perfect match to the sequences of the primers and probes used in the assay. The small sequence differences between the sense primer and target (one mismatch with published type 2 sequences and two mismatches with type 3 sequences [26]) could potentially have a large impact upon the relative amounts of sequences transcribed during the exponential phase of amplification (29). Although the in-house limiting dilution assay uses primers with similar mismatches, quantitation is unlikely to be affected to the same extent because the target cDNA sequences are isolated by dilution prior to PCR, and so are amplified without competition from other sequences. Since nested PCR produces large amounts of product from as little as a single input DNA copy, minor differences in amplification efficiency resulting from mismatches are unlikely to influence whether sequences isolated at limiting dilution can be detected.

Virus load and genotype. Previous studies with the bDNA-1 assay, Roche Monitor assay, and competitive PCR have all identified significantly higher levels of viremia in type 1 patients than in type 2 or 3 patients prior to interferon therapy (2, 3, 10, 12, 17). On the basis of these results, it has been proposed that this difference in viral titers accounts, at least in part, for the poor response to interferon treatment of type 1-infected patients. However, the results of the current study concur with more recent data based on the use of corrected bDNA-1 assay values (13, 25) which found no significant difference in virus load among blood donors or patients infected with different genotypes. These findings have the important implication that genotype and virus load must be independent predictors of the response to interferon (16, 28).

The findings also suggest that there are no major differences between genotypes in replication rate or susceptibility to immune clearance in untreated patients. This finding is consistent with previous observations that multiply exposed individuals, such as hemophiliacs, do not become preferentially infected with one particular genotype (11). A predominance of type 1 infections in hemophiliacs would be expected if this genotype replicated to a significantly higher level in vivo.

In summary, we have provided evidence that levels of circulatory viremia in type 1, 2, and 3 infections may be quantified with unequal efficiencies and that the magnitude of this difference in the Roche Monitor assay is likely to have significant, confounding effects upon statistical analyses of virus load and genotype. There is clearly a need to more accurately measure the difference in sensitivity for a wider range of genotypes, including type 4, and to apply the resulting correction factors to the results of the large number of completed or ongoing clinical studies in which virus load measurements have been based upon this method. In the future, it may be possible to correct the primer-target mismatches in the Roche Monitor assay if that is the cause of the unequal quantitation and therefore provide a more satisfactory assay for clinical studies.

ACKNOWLEDGMENTS

We are grateful to the staff at Scottish National Blood Transfusion Service Microbiology Reference Laboratory for providing plasma samples and to J. Detmer and J. Kolberg for providing RNA transcripts of genotypes 1, 2, and 3.

This study was funded in part by a research grant to A.H. and P.S. from Nucleic Acid Systems, Chiron Corporation.

REFERENCES

1. **Arase, Y., H. Kumada, K. Chayama, M. Naoya, A. Tsubota, I. Koida, M. Kobayashi, Y. Suzuki, K. Ikeda, and S. Saitoh.** 1995. Usefulness of HCV-RNA counts by the method of HCV-bDNA probe in interferon retreatment for patients with HCV-RNA positive chronic hepatitis C. Int. Hepatol. Commun. **4:**19–25.
2. **Berger, A., M. vonDepkaProndzinski, H. W. Doerr, H. Rabenau, and B. Weber.** 1996. Hepatitis C plasma viral load is associated with HCV genotype but not with HIV coinfection. J. Med. Virol. **48:**339–343.
3. **Chan, C. Y., S. D. Lee, S. J. Hwang, R. H. Lu, C. L. Lu, and K. J. Lo.** 1995. Quantitative branched DNA assay and genotyping for hepatitis C virus RNA in Chinese patients with acute and chronic hepatitis C. J. Infect. Dis. **171:** 443–446.
4. **Chan, S.-W., F. McOmish, E. C. Holmes, B. Dow, J. F. Peutherer, E. Follett, P. L. Yap, and P. Simmonds.** 1992. Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. J. Gen. Virol. **73:**1131–1141.
5. **Collins, M. L., C. Zayati, J. J. Detmer, B. Daly, J. A. Kolberg, T. A. Cha, B. D. Irvine, J. Tucker, and M. S. Urdea.** 1995. Preparation and characterization of RNA standards for use in quantitative branched DNA hybridization assays. Anal. Biochem. **226:**120–129.
6. **Daly, J. A., and G. Ertingshausen.** 1972. Direct method for determining inorganic phosphate in serum with the "CentrifiChem." Clin. Chem. **18:**263–265.
7. **Davidson, F., P. Simmonds, J. C. Ferguson, L. M. Jarvis, B. C. Dow, E. A. C. Follett, C. R. G. Seed, T. Krusius, C. Lin, G. A. Medgyesi, H. Kiyokawa, G. Olim, G. Duraisamy, T. Cuypers, A. A. Saeed, D. Teo, J. Conradie, M. C. Kew, M. Lin, C. Nuchaprayoon, O. K. Ndimbie, and P. L. Yap.** 1995. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5′ non-coding region. J. Gen. Virol. **76:**1197–1204.
8. **Davis, G. L.** 1994. Prediction of response to interferon treatment of chronic hepatitis C. J. Hepatol. **21:**1–3.
9. **Detmer, J., R. Lagier, J. Flynn, C. Zayati, J. Kolberg, M. Collins, M. Urdea, and R. Sanchez-Pescador.** 1996. Accurate quantification of hepatitis C virus (HCV) RNA from all HCV genotypes by using branched-DNA technology. J. Clin. Microbiol. **34:**901–907.
10. **Hayashi, J., Y. Kishihara, E. Yoshimura, Y. Tani, K. Yamaji, H. Ikematsu, H. Ishiko, and S. Kashiwagi.** 1995. Relationship of genotype to level of hepatitis C viraemia determined by competitive polymerase chain reaction. J. Infect. **30:**235–239.
11. **Jarvis, L. M., C. A. Ludlam, J. A. Ellender, L. Nemes, S. P. Field, E. Song, A. Chuansumrit, F. E. Preston, and P. Simmonds.** 1996. Investigation of the relative infectivity and pathogenicity of different hepatitis C virus genotypes in hemophiliacs. Blood **87:**3007–3011.
12. **Lau, J. Y. N., G. L. Davis, J. Kniffen, K. P. Qian, M. S. Urdea, C. S. Chan, M. Mizokami, P. D. Neuwald, and J. C. Wilber.** 1993. Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet **341:**1501–1504.
13. **Lau, J. Y. N., G. L. Davis, L. E. Prescott, G. Maertens, K. L. Lindsay, K. P. Qian, M. Mizokami, P. Simmonds, R. P. Perrillo, E. R. Schiff, H. C. Bodenheimer, L. A. Balart, F. Regenstein, J. L. Dienstag, W. N. Katkov, C. H. Tamburro, J. S. Goff, G. T. Everson, Z. Goodman, and J. Albrecht.** 1996. Distribution of hepatitis C virus genotypes determined by line probe assay in patients with chronic hepatitis C seen at tertiary referral centers in the United States. Ann. Intern. Med. **124:**868.
14. **Lau, J. Y. N., P. Simmonds, and M. S. Urdea.** 1995. Implications of variations of "conserved" regions of hepatitis C virus genome. Lancet **346:**425–426.
15. **Mahaney, K., V. Tedeschi, G. Maertens, A. M. Dibisceglie, J. Vergalla, J. H. Hoofnagle, and R. Sallie.** 1994. Genotypic analysis of hepatitis C virus in American patients. Hepatology **20:**1405–1411.
16. **Martinot Peignoux, M., P. Marcellin, M. Pouteau, C. Castelnau, N. Boyer, M. Poliquin, C. Degott, I. Descombes, V. Lebreton, V. Milotova, J. P. Benhamou, and S. Erlinger.** 1995. Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. Hepatology **22:**1050–1056.
17. **Matsumoto, A., E. Tanaka, T. Suzuki, H. Ogata, and K. Koyosawa.** 1994. Viral and host factors that contribute to efficacy of interferon-alpha(2a) therapy in patients with chronic hepatitis C. Dig. Dis. Sci. **39:**1273–1280.
18. **McOmish, F., S.-W. Chan, B. C. Dow, J. Gillon, W. D. Frame, R. J. Crawford, P. L. Yap, E. A. C. Follett, and P. Simmonds.** 1993. Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serological reactivity and rate of alanine aminotransferase abnormalities. Transfusion **33:**7–13.
19. **Mita, E., N. Hayashi, H. Hagiwara, K. Ueda, Y. Kanazawa, A. Kasahara, H. Fusamoto, and T. Kamada.** 1994. Predicting interferon therapy efficacy from hepatitis C virus genotype and RNA titer. Dig. Dis. Sci. **39:**977–982.
20. **Mizokami, M., E. Orito, Y. Gibo, K. Suzuki, K. Ohba, T. Ohno, and J. Y. N. Lau.** 1996. Genotype, serum level of hepatitis C virus RNA and liver histology as predictors of response to interferon-alpha 2a therapy in Japanese patients with chronic hepatitis C. Liver **16:**23–27.
21. **Nousbaum, J. B., S. Pol, B. Nalpas, P. Landais, P. Berthelot, C. Brechot, M. Gigou, C. Feray, V. Thiers, H. Okamoto, S. Mishiro, K. Poussin, P. Paterlini, M. Rumi, and M. Colombo.** 1995. Hepatitis C virus type 1b (II) infection in France and Italy. Ann. Intern. Med. **122:**161.
22. **Orito, E., M. Mizokami, T. Nakano, H. Terashima, O. Nojiri, H. Sakakibara, M. Mizuno, M. Ogino, M. Nakamura, Y. Matsumoto, K. I. Miyata, and J. Y. N. Lan.** 1994. Serum hepatitis C virus RNA level as a predictor of subsequent response to interferon-alpha therapy in Japanese patients with chronic hepatitis C. J. Med. Virol. **44:**410–414.
23. **Simmonds, P., P. Balfe, J. F. Peutherer, C. A. Ludlam, J. O. Bishop, and A. J. L. Brown.** 1990. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J. Virol. **64:**864–872.
24. **Simmonds, P., L. Q. Zhang, H. G. Watson, S. Rebus, E. D. Ferguson, P. Balfe, G. H. Leadbetter, P. L. Yap, J. F. Peutherer, and C. A. Ludlam.** 1990. Hepatitis C quantification and sequencing in blood products, haemophiliacs, and drug users. Lancet **336:**1469–1472.
25. **Smith, D. B., F. Davidson, P. L. Yap, H. Brown, J. Kolberg, J. Detmer, M. Urdea, P. Simmonds, J. Mellor, J. Neville, L. Prescott, B. C. Dow, E. A. C. Follett, T. Skuldamrongpanich, S. Tanprasert, C. Nuchaprayoon, C. K. Lin, M. C. Kew, R. Crookes, J. D. Conradie, M. Lin, C. Seed, G. A. B. Deolim, I. A. Martins, G. A. Medgyesi, T. Krusius, A. A. Saeed, D. Teo, and S. H. Lee.** 1996. Levels of hepatitis C virus in blood donors infected with different viral genotypes. J. Infect. Dis. **173:**727–730.
26. **Smith, D. B., J. Mellor, L. M. Jarvis, F. Davidson, J. Kolberg, M. Urdea, P. L. Yap, P. Simmonds, J. D. Conradie, A. G. S. Neill, G. M. Dusheiko, M. C. Kew, R. Crookes, A. Koshy, C. K. Lin, C. Lai, I. M. Murraylyon, A. Elguneid, A. A. Gunaid, T. Yemen, S. Yemen, D. Mutimer, M. Ahmed, C. Nuchprayoon, S. Tanprasert, F. E. Preston, M. Makris, A. Chuansumrit, C. Mahasandana, D. Pritchard, E. Riley, B. M. Greenwood, A. A. Saeed, A. M. Alrasheed, M. G. Saleh, I. Mcfarlane, C. Tibbs, R. Williams, J. Power, E. Lawlor, and H. Kiyokawa.** 1995. Variation of the hepatitis C virus 5′ noncoding region: implications for secondary structure, virus detection and typing. J. Gen. Virol. **76:**1749–1761.
27. **Warshaw, M. M., and I. Tinico.** 1966. Optical properties of sixteen dinucleoside phosphates. J. Mol. Biol. **20:**29–38.
28. **Yamada, G., R. Takatani, F. Kishi, M. Takahashi, T. Doi, T. Tsuji, S. Shin, M. Tanno, M. S. Urdea, and J. A. Kolberg.** 1995. Efficacy of interferon alfa therapy in chronic hepatitis C patients depends primarily on hepatitis C virus RNA level. Hepatology **22:**1351–1354.
29. **Young, K. K. Y., R. M. Resnick, and T. W. Myers.** 1993. Detection of hepatitis C virus RNA by a combined reverse transcription-polymerase chain reaction assay. J. Clin. Microbiol. **31:**882–886.
30. **Zhang, L. Q., P. Simmonds, C. A. Ludlam, and A. J. Leigh Brown.** 1991. Detection, quantitation and sequencing of HIV-1 virus from the plasma of seropositive individuals and from factor VIII concentrates. AIDS **5:**675–681.